Characterization of Eosinophil Adhesion to TNF-α-Activated Endothelium Under Flow Conditions: α_4 Integrins Mediate Initial Attachment, and E-Selectin Mediates Rolling[1]

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The multistep model of leukocyte adhesion reveals that selectins mediate rolling interactions and that integrins mediate firm adhesion processes. In this study, the interaction between eosinophils and TNF-α-activated HUVEC (second or third passage) was studied under flow conditions (0.8 and 3.2 dynes/cm^2). Especially the role of α_4 integrins on eosinophils and E-selectin on HUVEC was studied. Inhibition of the integrin α_4 chain on eosinophils reduced the number of firmly adhered resting eosinophils to TNF-α-stimulated endothelium by 43% whereas the percentage rolling cells increased 2.2-fold compared with untreated control eosinophils. Blocking of E-selectin on the endothelium reduced the number of adherent eosinophils by only 23% and 16%. In this situation, however, hardly any rolling adhesion was observed, and the few rolling cells showed a low rolling velocity. Blocking both α_4 integrin on eosinophils and E-selectin on HUVEC reduced the number of adhered eosinophils by 95%. P-selectin did not significantly participate in eosinophil adhesion to TNF-α-activated HUVEC. Inhibition of both α_4 integrins and β_2 integrins on eosinophils resulted in a reduction of adhered cells by 65% and a 3-fold increase in percentage rolling cells. Taken together, these results clearly show that resting eosinophils preferentially use constitutively active α_4 integrins ($\alpha_4\beta_1$, $\alpha_4\beta_7$) for the first attachment to TNF-α-activated HUVEC. In addition, α_4 integrins and E-selectin work synergistically in eosinophil adherence to TNF-α-activated HUVEC. Although E-selectin is important for eosinophil rolling under these conditions, P-selectin plays only a minor role. *The Journal of Immunology,* **1999, 163: 343–350.**

Eosinophils play an important role in allergic inflammatory diseases such as allergic asthma. Infiltrates of these cells are present in the interstitium and the lumen of the bronchi of asthmatic patients (1). Eosinophils have to pass the endothelium to enter this site of inflammation. A widely accepted paradigm for leukocyte extravasation is the multistep model. In this model, selectins mediate rolling interactions between leukocytes and endothelium, and, subsequently, activated integrins facilitate firm adhesion and extravasation of the cells (2). For eosinophils these specific interactions with the endothelium are not fully elucidated and are the subject of this study.

In marked contrast to neutrophils, eosinophils constitutively express the β_1 integrin $\alpha_4\beta_1$ (very late Ag-4 (VLA-4),[3] CD49dCD29) (3–6). VLA-4 is also present on monocytes, lymphocytes, and basophils, whereas its counter structure VCAM-1 is present on activated endothelium (7, 8). Although selectins primarily mediate rolling interactions, it has been suggested that VLA-4 on lymphocytes (9, 10) and cell lines (11) can play a role in this process as well. For eosinophils, discrepancies exist on the role of α_4 integrin in mediating initial attachment to the endothelium. A study in postcapillary venules of IL-1β-activated rabbit mesentery showed that eosinophils utilize VLA-4 and L-selectin for rolling interactions. However, still 50% of the rolling interactions persisted even though VLA-4 and L-selectin were blocked (12). Very recently, Patel et al. (13) showed that eosinophils use α_4 integrins in tethering to IL-4-stimulated endothelial cells under flow conditions. In contrast, Kitayama et al. showed in a flow chamber model that α_4 integrins did not mediate initial attachment nor constitutive rolling interactions of eosinophils on TNF-α-activated HUVEC but only mediated immediate arrest after P-selectin-dependent initial attachment (14).

In contrast to α_4 integrins, E-, P-, and L-selectin are generally agreed upon as very important molecules in initial tethering and rolling adhesion of leukocytes to endothelium, including eosinophils. Under static conditions, eosinophils can adhere to both P- and E-selectin (4, 15). Under flow conditions, eosinophils have been reported to accumulate more avidly on P-selectin compared with neutrophils (16). On the other hand, neutrophils adhere more efficiently to E-selectin-coated surfaces under flow conditions (17). In concordance with these findings, Kitayama et al. showed a role for P-selectin in the first attachment of eosinophils to TNF-α-stimulated first passage HUVEC and did not see an effect on primary tethering when E-selectin was blocked (14). Taken together, eosinophils seem to be less E-selectin dependent for rolling interactions with activated endothelium when compared with neutrophils. The role of L-selectin in rolling of eosinophils is thought to be of less importance than E- and P-selectin. However, L-selectin has been shown to mediate adhesion of eosinophils to HUVEC under conditions of shear although experiments were performed at 4°C and no controlled shear stress was used (18).

Increased expression of VCAM-1, E-selectin, and ICAM-1 is associated with pulmonary allergic inflammation (19–22).

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Received for publication November 20, 1998. Accepted for publication April 7, 1999.

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[1] This work was supported by a grant from the Dutch Asthma Foundation (Nederlands Astma Fonds), Project Number 96.49.

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[3] Abbreviations used in this paper: VLA-4, very late Ag-4; PSGL-1, P-selectin glycoprotein ligand-1; HSA, human serum albumin.

0022-1767/99/$02.00

Therefore, these molecules and their ligands might be adhesion receptors for eosinophils to adhere to postcapillary venules present in the bronchial mucosa. However, many animal models do not lead to a consensus regarding this issue. An interesting primate model for asthma showed that anti-ICAM-1 therapy reduces lung eosinophilia and hyperreactivity (23). In addition, these authors showed that, after single Ag exposure, anti-E-selectin treatment reduces lung neutrophilia in primates. In rats, anti-VLA-4 treatment did not have an effect on neutrophilia (24) or eosinophilia (25). In contrast, the combination treatment of anti-α_4 integrins and anti-VCAM-1 showed a reduction in eosinophil and lymphocyte infiltration in the lung of mice (26). In vivo studies in mice showed that recruitment of eosinophils (27, 28) and other leukocytes (29) at inflammatory sites is mainly mediated by P-selectin. However, these studies also suggest a role for E-selectin in this process (28, 29).

In the current study the interactions between eosinophils and TNF-α-activated HUVEC were investigated in an in vitro flow chamber model. Special emphasis was given to α_4 integrins on the eosinophils and E-selectin on the endothelium.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). fMLP was purchased from Sigma (St. Louis, MO). Human serum albumin (HSA) was purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Recombinant human TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany). HEPES incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) HSA. All other materials were reagent grade.

Antibodies

The mAb HP2/1 (anti-VLA-4, CD49d) was purchased from Immunotech (Marseille, France). Two blocking anti-E-selectin (CD62e) mAbs were used; ENA_2 was kindly provided by Dr. W. A. Buurman (University Hospital, Maastricht, The Netherlands) (30), and BBIG-E4 (5D11) was purchased from R&D Systems (Abingdon, U.K.). mAb IB4 and mAb DREG56 were isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (ATCC, Manassas, VA). The mAb WASP12.2 (anti-P-selectin, CD62p) (31) was purchased from Endogen (Boston, MA). The mAb PL-1 (anti-P-selectin glycoprotein ligand (PSGL)-1, anti-CD 162) was purchased from Immunotech. All above mentioned mAbs are functionally blocking Abs. Control Ab W6/32 (anti-HLA-A,-B,-C) was isolated from the supernatant of a hybridoma obtained from ATCC. mAbs were incubated with eosinophils (4×10^6 cells/ml) or with confluent HUVEC layers on cover slips at 10 μg/ml during 15 min before the experiments. The cell suspensions were diluted twice with incubation buffer (final concentration of 5 μg/ml mAb at 2×10^6 cells/ml in HEPES incubation buffer), and the coverslips were placed directly in the system.

Isolation of eosinophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy coat of 500 ml blood anticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4) as previously described (32). Mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of the erythrocytes with an isotonic ice cold NH_4Cl solution, the granulocytes were washed and resuspended in RPMI 1640 (Life Technologies, Paisley, U.K.) with 0.5% (w/v) HSA.

Granulocytes were incubated for 30 min at 37°C to restore the initial density of the cells. Thereafter, the cells were washed and resuspended in PBS supplemented with 0.5% HSA and 13 mM trisodium citrate, and incubated with fMLP (10 nM) for 10 min at 37°C, to decrease the specific gravity of the neutrophils, but not that of the eosinophils. Subsequently, eosinophils were obtained by centrifugation (20 min, $1000 \times g$) over isotonic Percoll (density 1.082 g/ml, layered on percoll with a density of 1.1 g/ml), washed, and resuspended in HEPES incubation buffer. Purity of eosinophils was >95%, (and recovery was usually 80–90%). This procedure leads to the isolation of relatively unprimed eosinophils compared with conventionally used isolation procedures with immunomagnetic beads (33).

Endothelial cells

HUVEC were isolated from human umbilical cord veins according to Jaffe et al. (34), with some minor modifications (35). The cells were cultured in RPMI 1640 containing 20% (v/v) heat-inactivated human serum, 200 μg/ml penicillin/streptomycin (Life Technologies, Breda, The Netherlands), and fungizone (Life Technologies). Cell monolayers were grown to confluence in 5–7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVEC was activated by TNF-α (100 U/ml, 7 h, 37°C) before the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber (36) as previously described by Van Zanten et al. (35). This microchamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm × 18 mm) with confluent HUVEC was mounted.

Eosinophil perfusion and evaluation

Eosinophils in suspension (2×10^6 cells/ml in HEPES incubation buffer) were aspirated from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natick, MA). In this way, the flow rate through the chamber could be precisely controlled. The wall shear stress (t) was calculated according to the Navier Stokes equation: $t = (6Q \cdot \eta)/(w \cdot h^2)$. In this equation Q is the volumetric flow rate, η is the suspending medium coefficient of viscosity (assumed to be equal to water at 0.01 poise), w is the slit width, and h is the slit height. The shear stress is proportional to the rate of flow of the cells and can be calculated as dynes/cm^2.

Eosinophil perfusions were performed as individual runs under specific shear conditions all in a 37°C temperature box. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany) that was equipped with a black and white CCD video camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adhered cells, the rolling velocity per cell, and the cluster index, using dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silver Spring, MD). The eosinophils that were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adhering cells on the HUVEC were detected by the image analyzer. The number of surface-adhered eosinophils was measured after 5 min perfusion at a minimum of 25 randomized high power fields (total surface of at least 1 mm^2). Then buffer was perfused, and shear rates were increased from 0.8 to 2, 3.2, and 6.4 dynes/cm^2 each for 1 min, during which high power fields were recorded to determine rolling velocities at these shear rates. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (δt, with a minimal interval of 80 milliseconds) was digitally captured. At each frame, the position of every cell was detected, and, for all subsequent frames, the distance moved by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell (v) in micrometers per sec was calculated from the equation: $v = L/\delta t(x\text{-}1)$ in which L is the covered distance (μm), δt is the time interval between images (s), and x is the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/s. Rolling velocity classes were depicted, which is defined as the fraction of the total number of rolling cells in each group exhibiting a rolling velocity corresponding to the velocity classes as indicated (see Fig. 3). With this method, static adherent, rolling, and freely flowing cells (which were not in focus) could be clearly distinguished. Cluster index was measured as previously described (37). The number of surface-adhered eosinophils per mm^2 was measured after 5 min of perfusion at a minimum of 20 randomized fields. For each adherent cell the number of cells in the surrounding area of ~1750 μm^2 was measured. In the case of a random distribution, the expected number of cells inside this area was calculated based upon the mean number of surface-adherent cells per mm^2. The cluster index, set to be the difference between the measured and the expected number of cells inside an arbitrary area around the cell, was calculated using the following equation: cluster index per cell $= |m - [(X * a)/(A - 1)]|$ in which m is measured number of cells in the rectangle area, X is the total number of cells in the image, A is the size of the total image, and a is the size of a rectangular cell-surrounding area. For each experiment the mean cluster index of a minimum of 500 cells was calculated.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis of the data was performed using a one-way ANOVA against fixed value with Bonferroni correction, a one-way ANOVA with Bonferroni correction, or paired Student *t* test, as indicated in the figures. *P* values ≤ 0.05 were considered to be significant.

Results

E-selectin and α_4 integrins are important adhesion molecules in initial attachment to TNF-α-stimulated HUVEC

The role of different adhesion molecules in eosinophil-endothelium interactions was examined using blocking Abs. Fig. 1 shows the number of adhering cells per mm^2 (which equals the total number of firmly adhered and rolling cells) to activated HUVEC (TNF-α, 100 U/ml, 7 h) under flow conditions (0.8 dynes/cm^2) as a percentage of control (untreated) eosinophils. Fig. 1*A* focuses on the adhesion molecules mediating rolling, and Fig. 1*B* focuses on those mediating adhesion interactions. Treatment of eosinophils with the anti-PSGL-1 Ab PL-1 or treatment of HUVEC with anti-P-selectin WASP12.2 did not significantly decrease the number of adherent cells, showing the minor importance of these adhesion molecules under these conditions. HUVEC incubated with anti-E-selectin Abs (ENA-2 or 5D11) showed a slight decrease in adherent cells. However, this did not reach significance. When eosinophils were incubated with the anti-integrin α_4 chain Ab HP2/1 only 58 ± 7% of the eosinophils were able to adhere. Interestingly, a combination of anti-α_4 mAb with anti-E-selectin mAbs ENA-2 or BBA2 almost completely abolished adhesion. The percentage of binding eosinophils was only 4.3 ± 1.6% and 5.9 ± 1.7%, respectively. This additive effect was not seen for anti-P-selectin treatment in combination with HP2/1 (46 ± 16% adhesion) (Fig. 1*A*). In Fig. 1*B*, the treatment of eosinophils with the anti-integrin α_4 chain Ab HP2/1 is shown again. Furthermore, the combination of blocking α_4 integrins and β_2 integrins resulted in an additive effect whereby 35 ± 9% of the cells stayed adherent. This effect was significant (paired Student *t* test; $p = 0.002$). The combination of blocking E-selectin and α_4 integrins is significantly different from blocking α_4 integrins alone (ANOVA with Bonferroni correction: $p < 0.005$). Treatment of anti-β_2 integrins by mAb IB4 resulted in 89 ± 7% of adhering eosinophils compared with control. Also, blocking both β_2 integrins on the eosinophils and E-selectin on the endothelium did not result in a change in the percentage of adherent cells (89 ± 12%). Treatment of eosinophils with the Ab W6/32 (anti-HLA-A, -B, and -C) compared with control eosinophils (no mAb) showed no difference in adhesion, 1169 ± 233 and 1292 ± 134, respectively ($p = 0.362$, paired Student *t* test).

E-selectin, α_4 integrins, and β_2 integrins function differently in the rolling behavior of eosinophils

The role of different adhesion molecules in rolling processes of eosinophils on activated endothelium was examined using blocking Abs. The percentage of rolling cells was determined by image analysis of video images (see *Materials and Methods*) (Fig. 2). Fig. 2*A* focuses on adhesion molecules mediating rolling interactions. On activated HUVEC (TNF-α, 7 h, 37°C), 21 ± 4.4% of the surface interacting eosinophils were rolling. Blocking PSGL-1 or P-selectin did not affect percentage rolling compared with the control situation. By blocking E-selectin, the percentage of rolling cells decreased by more then 50% compared with control (ENA-2, 4.2 ± 1.8%; BBA2, 8.0 ± 2.0%). A significant increase in the percentage of rolling cells was seen when α_4 integrins were blocked (44 ± 5.9%). However, blocking both α_4 integrins and P-selectin did not significantly differ from blocking α_4 integrins alone (54 ± 7.5%).

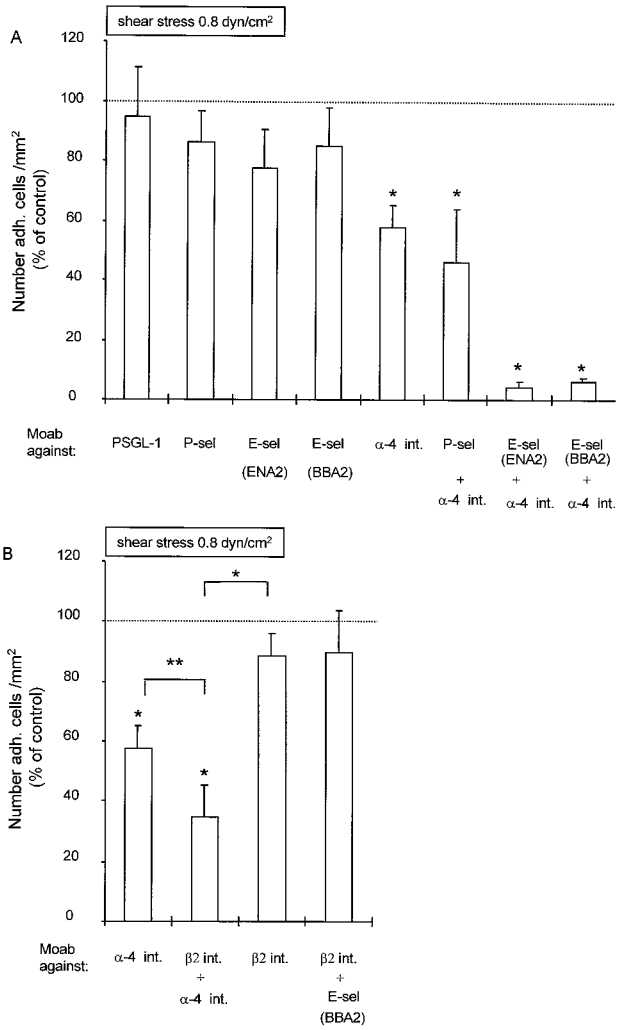

FIGURE 1. Effect of inhibition of selectins and integrins (int.) with blocking Abs on the interaction of eosinophils to TNF-α-activated endothelium. *A*, Shows the effect of blocking rolling receptors, and *B* shows the effect of blocking firm adhesion receptors (except the combination in which E-selectin is blocked). Cells (2×10^6/ml) preincubated in the presence or absence of blocking mAbs (10 μg/ml, 15 min, 37°C) were perfused at shear stress 0.8 dynes/cm^2 during 5 min, and images were recorded on video. At least 30 images were counted off line by image analysis (see *Materials and Methods*). The number of adhering eosinophils (rolling and firm adhering cells) was determined as a percentage of control values (resting, nontreated eosinophils on 7-h TNF-α-stimulated HUVEC). The absolute number of adhering cells in the control situation was 1142 ± 167 eosinophils/mm^2. Percentages are shown ± SEM of three to five different experiments. The statistically significant effects of the blocking Abs were determined by ANOVA against fixed value with Bonferroni correction for nonpaired data (*, $p < 0.001$) or by paired Student *t* test for paired data (**, $p = 0.002$).

Fig. 2*B* focuses on adhesion molecules mediating firm adhesion. The control situation and treatment of eosinophils by anti-α_4-integrins are depicted as in Fig. 1*A*. Blocking β_2 integrins did not have a significant effect on the percentage of rolling cells. By blocking both β_2 and α_4, again a significant increase in the percentage of rolling cells was seen (61 ± 8.1%). Simultaneously blocking α_4 integrins and E-selectin resulted in a too low amount of cells to evaluate the percentage of rolling cells.

Rolling velocities are shown in Fig. 3. The percentage of cells in each velocity class is plotted against the different velocity classes. Rolling velocity classes are defined as the fraction of the total

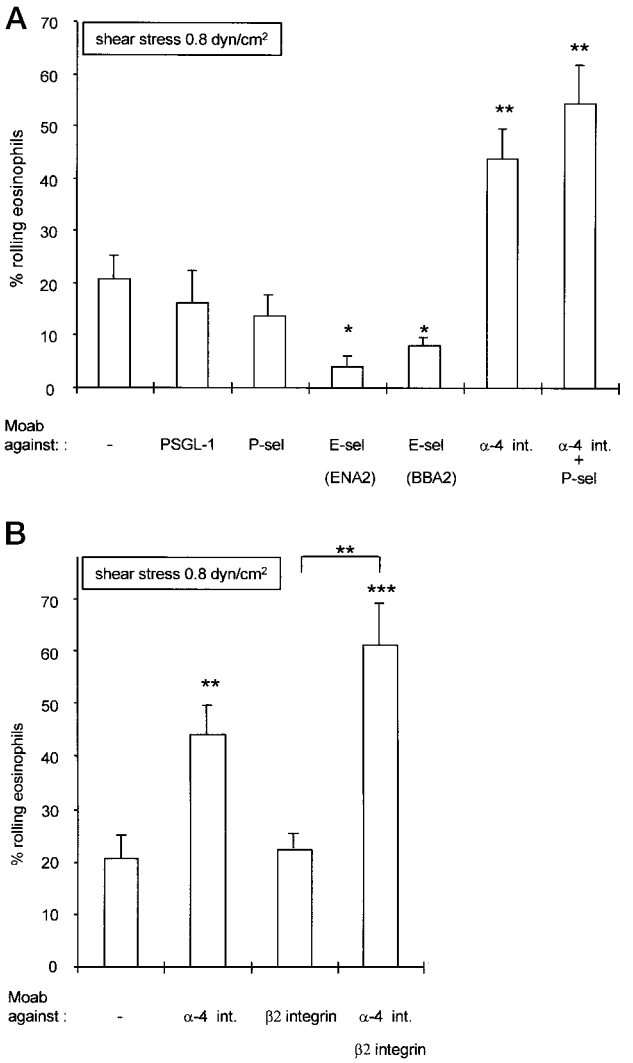

FIGURE 2. Effect of inhibition of selectins and integrins (int.) with blocking Abs on the percentage of rolling eosinophils on TNF-α-activated endothelium. *A*, Shows the effect of blocking rolling receptors, and *B* shows the effect of blocking firm adhesion receptors. Cells (2×10^6/ml) preincubated in the presence or absence of blocking mAbs (10 μg/ml, 15 min, 37°C) were perfused at shear stress 0.8 dynes/cm^2, and, during the second and third minute of perfusion, at least three areas were recorded on video for a period of at least 5 s. At least 100 cells were studied per perfusion, and data were analyzed off line by image analysis (see *Materials and Methods*). The percentage of rolling cells (>1 μm/s) of the total number of adhering cells was calculated. Means are plotted for three to four experiments ± SEM. The statistically significant effects of the blocking Abs against control situation or between different treatments (as indicated in the figure) were determined by paired Student *t* test (*, $p < 0.05$) or one-way ANOVA with Bonferroni correction (**, $p < 0.005$; ***, $p < 0.001$).

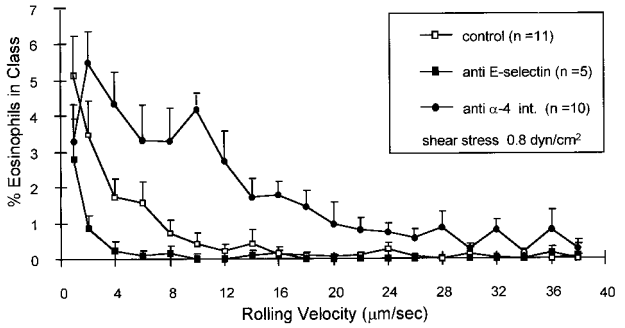

FIGURE 3. Effect of inhibition of selectins and integrins with blocking Abs on the rolling velocity of eosinophils on TNF-α-activated endothelium. Frequency distribution of rolling cells at shear stress 0.8 dynes/cm^2. The percentage of rolling cells of the total number of cells (total = rolling plus firmly adhering to the endothelium) in each velocity class is plotted. The sum of the percentages per group equals the percentages shown in Fig. 2. The first point represents velocity class of 1 to 2 microns/s; following points represent the velocity class of 2 to 4, 4 to 6 microns/s, etc. Cells rolling faster than 1 μm/s were considered as rolling cells. Data represent mean percentages ± SEM of the indicated number of experiments.

number of rolling cells in each group exhibiting a rolling velocity corresponding to the velocity classes as indicated. The median and mean rolling velocities are shown in Table I. The mean rolling velocity of control eosinophils to activated endothelium was 4.8 μm/s (median, 2.6 μm/s). Eosinophils treated with anti-PSGL-1 (PL-1; median, 2.8 μm/s), anti-β_2 (IB4; median, 3.1 μm/s), and HUVEC treated with WASP12.2 (anti-P-selectin; median, 3.6 μm/s) showed the same profile in frequency distribution as control cells (data not shown). However, when E-selectin was blocked, the mean rolling velocity decreased to 4.3 μm/s, and the median rolling velocity was shifted to 1.7 μm/s, although this was not significantly different from the control situation. By blocking α_4 integrins, it was shown that, besides the significant increase in the percentage of rolling cells, the mean rolling velocity of the cells increased to 12.2 μm/s (median, 9.4 μm/s). Blocking both α_4 integrins and P-selectin or both α_4 integrins and β_2 integrins resulted in similar velocity profiles, compared with blocking of α_4 integrins alone (data not shown; medians, 12.2 and 10.0 μm/s, respectively).

Effect of different shear rates on rolling velocities and behavior of eosinophils

The effects of different shear rates on median rolling velocities of resting untreated eosinophils and HP2/1-treated eosinophils were examined. Eosinophils were allowed to interact with the endothelial surface for 5 min (0.8 dynes/cm^2). Then buffer was perfused with increasing shear stresses of 2, 3.2, and 6.4 dynes/cm^2 each for 1 min. Rolling velocities of at least 100 cells were measured at these time intervals, and the median rolling velocity of the total number of rolling cells is shown in Table II. Increasing shear forces of 0.8, 2, 3.2, and 6.4 dynes/cm^2 had a slight increasing

Table I. *Effect of inhibition of selectins and integrins with blocking Abs on the median rolling velocity of eosinophils on TNF-α-activated endothelium*[a]

Blocking:	Median Rolling Velocity (μm/s)	Mean Rolling Velocity (μm/s)	*p* Value
Control	2.6	4.8	
P-selectin	3.6	5.2	NS
PSGL-1	2.8	6.1	NS
E-selectin	1.7	4.3	NS
VLA-4 (α_4 integrin)	9.4	12.2	<0.001
VLA-4 and P-selectin	12.2	14.4	<0.001
VLA-4 and β_2 integrins	10.0	12.7	<0.001
β_2 integrins	3.1	5.5	NS

[a] Eosinophils were incubated with indicated mAbs (10 μg/ml) for 15 min, and flow experiments on 7-h TNF-α-activated HUVEC at 0.8 dynes/cm^2 were performed. The rolling velocities of at least 100 cells per experiment out of at least three experiments were determined (see *Materials and Methods*), and the median and mean rolling velocities (μm/sec) were calculated. The statistically significant effects (*p*) of the blocking Abs against control situation were determined by one-way ANOVA with Bonferroni correction.

Table II. *Median rolling velocities and mean rolling velocities (± SEM) of total number of rolling cells at different shear rates for resting control eosinophils and anti-α_4 integrin-treated eosinophils on 7-h TNF-α-stimulated HUVEC*[a]

	Median (μm/s)		Mean (μm/s ± SD)		
dynes/cm^2	–	HP2/1	–	HP2/1	p Value
0.8	3.2	8.6	4.7 ± 0.34	10.9 ± 0.54	<0.001
2.0	3.8	12.8*	5.5 ± 0.24	13.6 ± 0.41[b]	<0.001
3.2	4.3	15.0	5.8 ± 0.27[b]	16.3 ± 0.41[b]	<0.001
6.4	5.8	19.5	8.0 ± 0.30[b]	21.0 ± 0.43[b]	<0.001

[a] Effect of different shear stresses on the rolling velocity of eosinophils on TNF-α-activated HUVEC in the presence or absence of an anti-α_4 integrin mAb (HP2/1). After 5 min of perfusion at shear stress 0.8 dynes/cm^2, the shear was increased to 2.0, 3.2, and 6.4 dynes/cm^2 during 1 min, respectively. Median and mean rolling velocities (μm/s) ± SEM are calculated from at least 100 cells (see *Materials and Methods*) out of three experiments (except *, $n = 2$). The statistically significant effects (p) of anti-α_4 integrin treatment (HP2/1) vs control situation were determined for each velocity class by one-way ANOVA.

[b] The significant effects ($p < 0.05$) of the mean rolling velocity in the shear class 2, 3.2, and 6.4 dynes/cm^2 vs the 0.8 dynes/cm^2 value in each group (control and HP2/1 treatment) determined by one-way ANOVA.

effect on median rolling velocity of resting eosinophils on TNF-α-activated HUVEC. The mean rolling velocity of control eosinophils at shear rates 3.2 and 6.4 dynes/cm^2 was significantly different ($p < 0.05$) from the rolling velocity at shear rate 0.8 dynes/cm^2. The median rolling velocity of eosinophils treated with HP2/1 increased from 8.6 μm/s at 0.8 dynes/cm^2 to 19.5 μm/s at 6.4 dynes/cm^2. These cells increased their mean rolling velocity with increasing shear stresses of 2, 3.2, and 6.4 dynes/cm^2 significantly ($p < 0.001$) compared with shear stress 0.8 dynes/cm^2. The mean rolling velocity of control eosinophils vs HP2/1-treated eosinophils was significantly lower at all four different shear rates ($p < 0.001$) (Table II).

Effect of high shear stress on adhesion, rolling, and rolling velocity of eosinophils

When resting, untreated eosinophils were perfused at shear stress 3.2 dynes/cm^2 during 5 min, the total number of adherent cells was 1089 ± 167, which was not significantly different from experiments done at shear stress 0.8 dynes/cm^2 ($p = 0.72$, paired Student t test). At shear stress 3.2 dynes/cm^2, strings (cell clusters in the direction of the flow) were visible. This suggests a role for L-selectin (19, 38). To address the role of L-selectin in this situation, blocking studies were performed. In the control situation, strings were observed (Fig. 4*A*), and the cluster index was 1.5 ± 0.2. Inhibition of L-selectin by blocking mAb DREG-56 (10 μg/ml) resulted in a cluster index of 0.85 ± 0.2 ($p = 0.05$, paired Student t test), and fewer strings were observed compared with the control situation as shown in Fig. 4*B*. Incubation of eosinophils with control Ab W6/32 (anti-HLA-A, -B, and -C) did not affect the percentage of adhering cells (103 ± 14%). Also, blocking E-selectin did not significantly influence the number of adhered cells (102 ± 14%) whereas blocking α_4 integrins did (39 ± 8%). When both L-selectin and E-selectin were blocked, still 30 ± 8% of the eosinophils stayed adhered (Table III). No cells were attached to the surface when both E-selectin and α_4 integrins were blocked, which is similar to the situation at shear stress 0.8 dynes/cm^2 ($n = 1$). The percentage of rolling cells at shear stress 3.2 dynes/cm^2 increased 1.5-fold compared with 0.8 dynes/cm^2. When α_4 integrins were blocked at shear stress 3.2 dynes/cm^2, 67.5 ± 14% of the cells were rolling. In contrast, when E-selectin was blocked, only 13.6 ± 6% of the cells showed a rolling interaction, which did not decrease further when L-selectin was blocked in addition to E-selectin. The mean rolling velocity in the control situation was 7 ± 0.5 μm/s, which is 1.5 times higher than at shear stress 0.8 dynes/cm^2. Inhibition of α_4 integrin increased the rolling velocity to 18 ± 0.6 μm/s, which is 1.6 times as high as at shear stress 0.8 (Table II). However, blocking E-selectin did not significantly reduce the rolling velocity compared with the control situation. Also, inhibition of both L-selectin and E-selectin did not significantly differ from the control situation.

Discussion

In this study, the interactions between resting human eosinophils and TNF-α-stimulated HUVEC were evaluated using a flow chamber model. TNF-α-stimulated HUVEC (7 h) was used to induce increased VCAM-1, E-selectin, and ICAM-1 expression, simulating the vasculature in allergic inflammation. The influence of inhibition of different adhesion molecules with blocking mAbs was evaluated with real time video-assisted image analysis. By determination of the number of adherent cells, the percentage of rolling cells, and rolling velocity distributions, we show that eosinophils

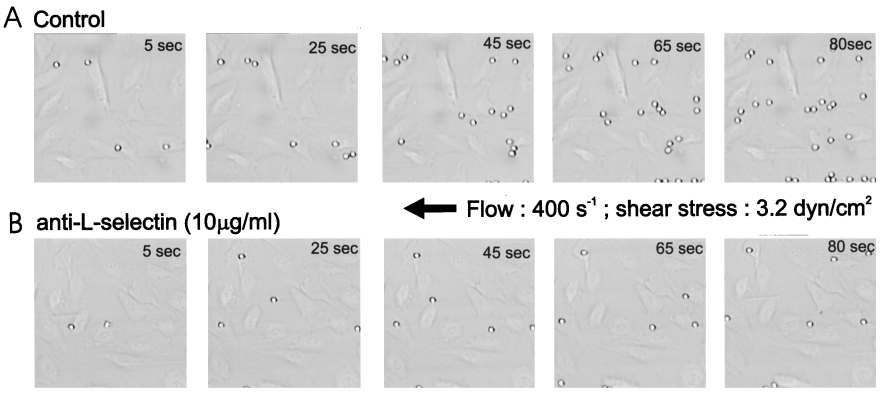

FIGURE 4. Effect of inhibition of L-selectin with blocking Ab DREG56 (10 μg/ml) on the distribution pattern of eosinophils on TNF-α-activated endothelium. Cells were perfused in the presence or absence of anti-L-selectin over 7-h TNF-α-activated HUVEC at shear stress 3.2 dynes/cm^2. Images were recorded on video during 5 min, and, subsequently, cluster indexes were determined (see *Materials and Methods*) (*A*, 1.8; *B*, 1.1). The images depicted above were taken after 30 s of cell perfusion in a time frame as indicated. The experiment shown is representative of two experiments.

Table III. *Effect of inhibition adhesion molecules with blocking Abs on adhesion, percentage of rolling cells, and the mean rolling velocity of eosinophils on TNF-α-activated endothelium at shear rate 3.2 dynes/cm²*[a]

Blocking:	Adherent Cells (% of control)	% Rolling Cells	Mean Rolling Velocity (μm/s)
Control	100	32.6 ± 8.5	7.1 ± 0.48
VLA-4 (α_4 integrin)	38.9 ± 7.7*	67.5 ± 14.2**	18.2 ± 0.64***
E-selectin	101.6 ± 13.6	13.6 ± 6.0**	7.3 ± 0.84
E-selectin and L-selectin	29.8 ± 7.6*	14.2 ± 3.4**	4.6 ± 0.69

[a] Eosinophils were incubated in the presence or absence of indicated mAbs (10 μg/ml) for 15 min, and flow experiments on 7-h TNF-α-activated HUVEC at 3.2 dynes/cm² were performed. Images were recorded on video. Data are plotted for three to seven experiments ± SEM. At least 30 images were counted off line by image, and number of adhering eosinophils (rolling and firm adhering cells) was determined as a percentage of control values (resting, nontreated eosinophils on 7-h TNF-α-stimulated HUVEC). The statistically significant effects of the blocking Abs were determined by ANOVA against fixed value with Bonferroni correction (*, $p < 0.005$). The percentage of rolling cells (> μm/s) of the total number of adhering cells was calculated. The statistically significant effects of Ab-treated eosinophils in contrast to control cells of percentage rolling cells were determined by a paired Student *t* test (**, $p < 0.05$). The rolling velocities of at least 40 cells per experiment were determined (see *Materials and Methods*). The statistically significant effects of Ab-treated eosinophils in contrast to control cells of mean rolling velocity were determined by one-way ANOVA with Bonferroni correction (***, $p < 0.001$).

preferentially use constitutively active α_4 integrins for mediating the first attachment to activated HUVEC. Also, E-selectin on the activated endothelium is used for initial attachment, and it mediates rolling interactions even at high shear stress. Most strikingly, blocking α_4 integrins and E-selectin together totally abolishes adhesion, indicating the strong cooperation of these two molecules in eosinophil adhesion to activated HUVEC and the minor importance of β_2 integrins under these conditions.

The well-established multistep paradigm for leukocyte-endothelium interactions describes rolling adhesion mediated by selectins and firm adhesion mediated by integrins. These processes are divided in time and place. However, the situation is more complex, since several recent studies describe a situation in which both tethering and firm adhesion are mediated by the same adhesion receptors. Clear examples for this overlapping behavior are the α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$), which have been described as rolling receptors (9–12), besides their role in firm adhesion (5, 14, 39). The α_4 integrins are present on lymphocytes, monocytes, basophils, and eosinophils but are not expressed on neutrophils. Berlin et al. (9) were the first to show α_4-dependent rolling of mouse lymph node cells on VCAM-1 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1)-coated surfaces. Later studies also showed initial tethering and rolling interactions by α_4 integrins of human T lymphocytes (11) and B lymphocytes (40) in a VCAM-1-dependent way. For monocytes, some studies show that α_4 integrins play a role in stabilizing initial tethering on activated endothelium (39, 41), and another study showed that monocytes can tether on VCAM-1 (42). For eosinophils, little is known about the role of α_4 integrins in attachment to inflamed endothelium. α_4 integrins on resting, nonactivated eosinophils are functionally active for adherence to VCAM-1-coated substrates or activated HUVEC under static conditions (3–6, 43). We confirmed this by performing experiments in which resting eosinophils bound to VCAM-1-coated magnetic beads or VCAM-1-coated 96-wells plates. This interaction could be specifically blocked by mAb HP2/1 (data not shown). However, the question remained whether α_4 integrins on human eosinophils are functionally active in mediating initial attachment to activated HUVEC under flow conditions. Thus far, a few groups have investigated this in an in vitro flow system (13, 14). We used an in vitro flow chamber assay to study the interactions of the α_4 integrins on eosinophils with activated endothelium under different shear forces. Also, the importance of the rolling receptors E- and P-selectin was studied, under the same conditions.

We found that inhibition of α_4 integrins (CD49d) by blocking anti-α_4 chain mAb HP2/1 resulted in more than a 40% decrease in the total number of adhering eosinophils to TNF-α-activated HUVEC (7 h, 100 U/ml) whereas blocking E-selectin or P-selectin did not have a significant effect on adhesion. Remarkably, by blocking both α_4 integrin and E-selectin the percentage of adhered eosinophils decreased by more than 95% (Fig. 1). This was found for each of the two anti-E-selectin Abs (ENA-2 and 5D11) that were tested. In contrast, no synergistic effect was seen when both α_4 integrins and P-selectin were blocked. We conclude that in our system both α_4 integrins and E-selectin are important for the first attachment to activated HUVEC at shear stress 0.8 dynes/cm². In contrast with another report (14), P-selectin does not play a role under these conditions (see below). Thus, α_4 integrins and E-selectin together are responsible for initial attachment and work synergistically in our model.

These results are in disagreement with the study of Kitayama et al. (14). These authors concluded that α_4 integrins play a minor role in initial attachment whereas we did find a major role for α_4 in this process. They also showed that P-selectin and not E-selectin is important in eosinophil rolling on TNF-α-stimulated HUVEC (6 h, 100 U/ml). These differences are difficult to explain. However, two considerations must be taken in account. 1) The (pre)activation status of eosinophils is of major importance in this type of studies. Eosinophils from mild eosinophilic donors (44) or cells isolated via different isolation procedures (33, 44) might have been preactivated and might have up-regulated their β_2 integrins and thereby have altered adhesion characteristics to endothelial cells (see below). 2) We used second passage HUVEC, which did not express an appreciable amount of P-selectin (data not shown), whereas Kitayama et al. used first passage HUVEC possibly expressing sufficient amounts of P-selectin (39, 42, 45). P-selectin can be expressed significantly on HUVEC in response to stimulation with histamine (46) or thrombin (31, 47). However, another important source for P-selectin is platelets. In this light, we recently showed that activated and P-selectin-expressing platelets can bind leukocytes, thereby facilitating P-selectin-dependent secondary tethering to the surface.[4] Interestingly, platelets have been

[4] P. H. M. Kuijper, J. J. Zwaginga, J. A. M. van der Linden, H. Gallardo-Torres, J.-W. J. Lammers, and L. Koenderman. Monocyte-associated platelets mediate P-selectin-dependent secondary tethering under flow conditions. *Submitted for publication.*

shown to adhere to eosinophils of allergic donors and might contribute to P-selectin-mediated interactions between eosinophils and endothelial cells (48). In our studies, eosinophils were carefully studied, and no attached platelets were present on the cell surface.

Blocking the β_2 integrins did not result in a significant decrease in adhesion whereas the combination of anti-α_4 and anti-β_2 resulted in a percentage of adherent cells of 35 ± 9% compared with the control situation (Fig. 1). This 65% reduction, significantly different from anti-α_4 treatment alone, suggests that β_2 integrins play only a minor role for resting eosinophil adherence to activated HUVEC. In contrast, Kitayama et al. (14) and Patel (13) showed that the combination of anti-β_2 integrins and anti-α_4 integrins totally abolished accumulation of eosinophils on 6-h TNF-α-activated HUVEC and 24-h IL-4-activated HUVEC, respectively. This suggests differences in the activating state of the β_2 integrins of eosinophils isolated by different methods (see above). The combination of blocking β_2 integrins and E-selectin did not have a significant effect on adhesion compared with control. This further suggests that α_4 integrins are perfectly capable of mediating initial attachment to the endothelium under these conditions.

Although eosinophils express less sialylated Lewis^x (Sle^x) than neutrophils, causing these cells to bind E-selectin less avidly (49), neutrophils as well as eosinophils are able to roll on E-selectin (50). Our results (Fig. 2) also show that eosinophils roll on E-selectin because inhibition of E-selectin results in immediate arrest of more than 90% of surface-interacting cells. This α_4-dependent immediate arrest was also shown by others for eosinophils (13, 14) and monoblastoid and lymphoblastoid cells (51). When α_4 integrins were blocked, however, eosinophils rolled on the surface using E-selectin. The anti-α_4-treated cells rolled faster compared with untreated control eosinophils. This suggests that the α_4 integrin/VCAM-1 interaction decreases cell rolling velocity. Simultaneous inhibition of α_4 and β_2 integrins did not result in higher rolling velocities (Fig. 3, Table I), thereby confirming the unprimed phenotype of eosinophils (52, 53). Blocking E-selectin reduced the rolling velocity of the eosinophils whereas PSGL-1 did not. This indicates that PSGL-1 is not the ligand for E-selectin on eosinophils. Alternatively, one (or more) of the known E-selectin ligands (cutaneous lymphocyte Ag (CLA) (54), E-selectin ligand 1 (ESL-1) (55), and/or L-selectin (56)) might be involved.

Next we wanted to test the resistance of the rolling interactions of eosinophils on activated HUVEC. Therefore, the shear stress was increased at the end of 5 min cell perfusion from 0.8 to 2, 3.2, and 6.4 dynes/cm^2 each for 1 min (Table II). Rolling velocities of resting eosinophils increased with increasing shear stresses (0.8 to 2, 3.2, and 6.4 dynes/cm^2) and a significant difference between mean rolling velocities was found between 0.8 and 6.4 dynes/cm^2. When these shear forces were applied to anti-α_4 integrin-treated eosinophils, cells rolled with rolling velocities that were at least two times higher at all four different shear stresses. These results again show that α_4 integrins are also active in reducing the rolling velocity at high shear stress.

At shear stress 0.8 dynes/cm^2, L-selectin played a minor role in eosinophil recruitment to the activated endothelium (data not shown). It is known that L-selectin is functionally important above a certain shear rate threshold (39, 42). To address the importance of L-selectin on eosinophils, which was shown to mediate adhesion to activated endothelium under shear (18), we applied 3.2 dynes/cm^2 to the system during 5 min (Fig. 4). L-selectin-dependent tethering with formation of cell clustering became important in the eosinophil recruitment to TNF-α-activated HUVEC at this shear stress. Inhibition of both α_4 integrins and E-selectin totally abolished adhesion of eosinophils, as was also seen at shear stress 0.8 dynes/cm^2, suggesting that L-selectin mediates only secondary tethering processes at high shear stresses. Remarkably, inhibition of both L-selectin and E-selectin resulted in a residual binding of 30 ± 8% of the eosinophils compared with the control situation (Table III). Thus, even at high shear stress, α_4 integrins are functional in mediating initial attachment to activated endothelium.

In conclusion, we show that resting eosinophils use constitutively active α_4 integrins and, to a lesser extent, E-selectin on TNF-α-activated HUVEC for initial attachment under flow conditions. Also, E-selectin can mediate stable rolling interactions even at high shear stresses. These data give new insights in the way eosinophils can get recruited to inflamed endothelium and emphasize the importance of E-selectin as a rolling receptor for eosinophils on TNF-α-activated endothelium. The importance of identification of the mechanisms involved in eosinophil extravasation is that it might lead to development of specific antagonists of this process, which might be used as targets for specific antiallergic treatment.

Acknowledgments

We thank Humberto Gallardo Torres for excellent technical and computer assistance.

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